AAC Journals.ASM.org

Effects of Zidovudine Treatment on Heart mRNA Expression and Mitochondrial DNA Copy Number Associated with Alterations in Deoxynucleoside Triphosphate Composition in a Neonatal Rat Model

Jacob W. Snowdin, Chia-Heng Hsiung, Daniel G. Kesterson, Vasudeva G. Kamath, Edward E. McKee

Foundational Sciences, College of Medicine, Central Michigan University, Mount Pleasant, Michigan, USA

The prevention of mother-to-child transmission (MTCT) of HIV is a crucial component in HIV therapy. Nucleoside reverse transcriptase inhibitors (NRTIs), primarily 3′-azido-3′-thymidine (AZT [zidovudine]), have been used to treat both mothers and neonates. While AZT is being replaced with less toxic drugs in treating mothers in MTCT prevention, it is still commonly used to treat neonates. Problems related to mitochondrial toxicity and potential mutagenesis associated with AZT treatment have been reported in treated cohorts. Yet little is known concerning the metabolism and potential toxicity of AZT on embryonic and neonatal tissues, especially considering that the enzymes of nucleoside metabolism change dramatically as many tissues convert from hyperplastic to hypertrophic growth during this period. AZT is known to inhibit thymidine phosphorylation and potentially alter deoxynucleoside triphosphate (dNTP) pools in adults. This study examines the effects of AZT on dNTP pools, mRNA expression of deoxynucleoside/deoxynucleotide metabolic enzymes, and mitochondrial DNA levels in a neonatal rat model. Results show that AZT treatment dramatically altered dNTP pools in the first 7 days of life after birth, which normalized to age-matched controls in the second and third weeks. Additionally, AZT treatment dramatically increased the mRNA levels of many enzymes involved in deoxynucleotide synthesis and mitochondrial biogenesis during the first week of life, which normalized to age-matched controls by the third week. These results were correlated with depletion of mitochondrial DNA noted in the second week. Taken together, results demonstrated that AZT treatment has a powerful effect on the deoxynucleotide synthesis pathways that may be associated with toxicity and mutagenesis.

Nucleoside reverse transcriptase inhibitors (NRTIs) are an important class of drug used primarily in treatment of the HIV infection and prevention of mother-to-child transmission (MTCT) of the virus. One of these NRTIs, 3′-azido-3′-thymidine (AZT [zidovudine]), is of interest as it is commonly used as a component of treatment for pregnant mothers and their newborn children. AZT was developed in 1974 as a cancer treatment drug, but in 1984 it was found to be more effective in treating HIV. Since then, it has been a major drug of choice used in highly active antiretroviral therapy (HAART) worldwide in adults, mothers, and their infants (1–3). While it is now being replaced with less toxic drugs, it is still commonly used to treat neonates (4, 5), in which AZT is highly efficient in preventing HIV transmission. AZT is given as a prodrug that must be phosphorylated by the host cell to AZT-triphosphate (AZT-TP), an analogue of TTP, in order to inhibit viral replication. While its metabolism and toxicity in adults have been well characterized, fetal and neonatal metabolism and toxicity have not been studied. Clinical manifestations, including suppression of bone marrow and anemia with low hemoglobin levels, have been reported in AIDS patients treated with AZT (6). Anemia and myelosuppression were also noted in non-infected infants of HIV-positive mothers who were treated with AZT (7). Additionally, AZT treatment has been shown to induce cardiotoxicity in children, as echocardiographs from a cohort of HIV-negative children revealed a significant decrease in left ventricular mass, left ventricular dimension, and septal wall thickness, which may impair myocardial growth (8). Eccentric left ventricle hypertrophy was seen in an AZT-treated HIV-1 transgenic mouse model (9). Transplacental exposure to AZT has been demonstrated to manifest cognitive impairments, including problems of spatial learning and memory in a mouse model (10). In addition to these clinical manifestations, changes in gene expression within the mitochondrial and nuclear genomes resulted from AZT treatment (11, 12). Significant reductions in oxidative phosphorylation and mitochondrial biogenesis were noted in cultured cardiomyocytes treated with AZT (12). In monkeys (13, 14), rats (15), mice (15, 16), and humans (17, 18), AZT has been shown to be mutagenic during the *in utero* and neonatal period. Phosphorylated AZT has been shown to competitively and noncompetitively inhibit mitochondrial DNA (mtDNA) polymerase-gamma, the enzyme responsible for mitochondrial DNA synthesis (19). Alternatively, AZT has been shown to inhibit thymidine kinase 2, preventing the synthesis of TTP in mitochondria of rat heart (20), liver (20), and brain (21) tissue, leading to potential depletion of TTP pools as was observed in the perfused heart (22). Endogenous thymidine serves as one of the essential precursors to mtDNA replication. Deoxynucleoside triphosphate (dNTP) asymmetries

Received 20 May 2015 **Returned for modification** 30 June 2015
Accepted 21 July 2015

Accepted manuscript posted online 27 July 2015

Citation Snowdin JW, Hsiung C-H, Kesterson DG, Kamath VG, McKee EE. 2015. Effects of zidovudine treatment on heart mRNA expression and mitochondrial DNA copy number associated with alterations in deoxynucleoside triphosphate composition in a neonatal rat model. Antimicrob Agents Chemother 59:6328–6336. doi:10.1128/AAC.01180-15.

Address correspondence to Edward E. McKee, mckee1ee@cmich.edu.

J.W.S. and C.-H.H. contributed equally to this paper.

doi:10.1128/AAC.01180-15

in mammalian mitochondria have been attributed to mutagenesis and reduced replication fidelity during DNA synthesis (23).

AZT has been widely used to prevent MTCT. The typical regimen began by treating mothers in their last trimester with oral AZT or Combivir (AZT and lamivudine) followed by intravenous AZT during the birth process. Finally, the neonates were treated with AZT during the first few months of life after birth. However, as noted above, concerns have been raised regarding possible toxic and mutagenic effects in infants treated with this AZT regimen. While AZT is being replaced in the treatment of mothers, it is still the recommended drug prescribed for neonates after birth (4). Despite the high use of this drug *in utero* and in neonates, its metabolism has never been studied under these circumstances and the mechanisms of its potential toxicity and mutagenesis are unknown.

During fetal development *in utero* and during the first week of life after birth, fetal/neonatal tissues such as the heart grow by cell division (hyperplasia). While growth continues, it changes to hypertrophic growth without cell division, as noted in adult tissue (24, 25). In cell cultures undergoing cell division, it is well known that dNTP levels are high and the enzyme pathways that make them are well expressed in order to replicate nuclear DNA. In contrast, the dNTP pools shrink dramatically in quiescent cultures that are not undergoing cell division but must still replicate their mtDNA. We hypothesize that the same events take place in the neonatal shift from hyperplastic to hypertrophic growth and that potential toxicity of AZT under these two conditions may be substantially different. During the hyperplastic growth period, NRTIs like AZT are likely to be phosphorylated at much higher levels than in adult tissues and may be associated with toxicities not noted in adults. As tissues convert to hypertrophic growth, the expression of enzymes of dNTP synthesis and salvage are thought to decline. AZT-TP made during the hyperplastic growth period may still be present as the cells enter hypertrophic growth. Concomitantly, the prodrug AZT is known to inhibit thymidine phosphorylation, lowering TTP levels. As a result, the boundary between hyperplastic and hypertrophic growth may be associated with an especially high ratio of AZT-TP to TTP, increasing the likelihood of mutagenesis in mtDNA. It is well known that imbalances in dNTP pools have been shown to give rise to defects in maintenance of mtDNA (26) and can be mutagenic in human and yeast cells (27) without the presence of AZT-TP. Finally, imbalances in the dNTP pools may alter gene expression in an attempt to correct the imbalance.

Considering the deleterious effects of AZT in the neonates noted above, we studied the effects of AZT on the composition and levels of dNTP pools and the expression of enzyme mRNAs responsible for dNTP synthesis in neonates as well as the ratio of mtDNA to nuclear DNA. This was accomplished by treating dams during the last week of pregnancy with AZT and continuing to treat the neonates at birth with AZT for 3 weeks. Results showed that dNTP pools were initially high during the first week of neonatal growth and decreased dramatically thereafter as tissues converted to hypertrophic growth. Consistent with that was a dramatic fall in the expression of mRNAs of many of the enzymes of dNTP synthesis and salvage. During this period, ratios of mitochondrial DNA to nuclear DNA doubled. AZT had profound effects on dNTP pool asymmetry and dramatically induced many of the enzymes of dNTP synthesis and salvage during the first week of life. These decreased to age-matched control levels by the third week of growth, even though AZT was still present.

(Portions of the data in this study were presented at the 4th Regional Translational Research in Mitochondria, Aging, and Disease Conference [Pittsburgh, PA], the Annual Student Research and Creative Endeavors Exhibition at Central Michigan University [Mount Pleasant, MI], and the Central Michigan University Capital Scholars Program [Lansing, MI].)

MATERIALS AND METHODS

Animal treatment. In-house outbred Harlan-Sprague-Dawley rats were used and handled as described under an Institutional Animal Care and Use Committee (IACUC)-approved protocol. Breeder male rats were paired with two female rats overnight for two nights. To confirm pregnancy, female rats were examined for sperm plug or sperm present in the vaginal smear daily. Once the pregnancy was confirmed, impregnated rats were weighed, separated, and marked as pregnant (gestation day 0). On gestation day 14 (~7 days before delivery), dams were given 100 mg AZT/kg body weight per day via drinking water until all the litters were weaned. Upon birth, daily treatment of the pups with 100 mg AZT/kg body weight in solution was initiated via oral gavage until sacrifice. Weights for both dams and pups were recorded daily. The animal treatment schedule corresponds to the treatment schedule in humans corrected for rat gestation and life span. The AZT dose was modeled after earlier rat and mouse studies (15, 28). While this is a suprapharmacological dose, it was done to limit the number of animals required for a proof-of-principle study. Neonatal rat pups were anesthetized according to IACUC guidelines by hypothermia on days −1, 1, and 3 and using a sodium pentobarbital solution (intraperitoneally [i.p.] 50 mg/kg body weight) on days 7, 14, and 21. Neonatal rats were euthanized 2 h after the final AZT treatment, and their major organs were harvested and immediately snap-frozen in liquid nitrogen and then stored at −80°C. Dams were also sacrificed in the same manner as that described above on the last day of their pregnancy, and both dam and fetal (day −1) organs were collected and snap-frozen in liquid nitrogen.

Tissue DNA and mRNA isolation. Snap-frozen fetal and neonatal tissues were powdered using a mortar and pestle chilled in liquid nitrogen and weighed for nucleic acid isolation. Genomic DNA was isolated using the DNA purification system (A2361; Promega), and total mRNA was isolated using the SV Total RNA isolation system kit (Z3101; Promega). Purity and quantity of the isolated RNA and DNA samples were analyzed using a UV spectrophotometer (NanoDrop; Thermo Scientific). The amount of mRNA/milligram of heart tissue as measured by optical density at 260 nm (OD_{260}) during the neonatal time course is shown in Fig. 1A. Total mRNA in the age-matched control hearts decreased by one-third by day 7. AZT treatment was associated with increased levels of mRNA/milligram of heart tissue, which peaked on day 7, nearly triple the age-matched control value ($P = 0.02$).

Preparation of tissue dNTPs. Snap-frozen fetal and neonatal tissues were collected as described in "Animal treatment," weighed, and homogenized in 5% trichloroacetic acid (TCA). Larger samples (>60 mg) from postgestation days 7 to 21 were powdered as described above prior to homogenization. Homogenates were centrifuged at 2,000 × *g* for 5 min at 4°C, and supernatants were transferred, neutralized with resin (AG-11A8; Bio-Rad), and filtered through a 0.45-μm nylon membrane. The final filtrate was used in the dNTP assay for quantification.

Quantification of tissue dNTPs. Template-driven DNA polymerase-based assays were performed using a protocol developed from Sherman and Fyfe (29) as modified by Mathews and Wheeler (30), except that the time of incubation was reduced to 3 min to minimize ribonucleotide incorporation (data not shown). [^{3}H]TTP or [^{3}H]dATP incorporation into the oligonucleotide templates was used to quantitate the desired dNTP level. Radioactivity was measured by a liquid scintillation counter (Tri-Carb 2800TR; PerkinElmer). Counts per minute (CPM) were con-

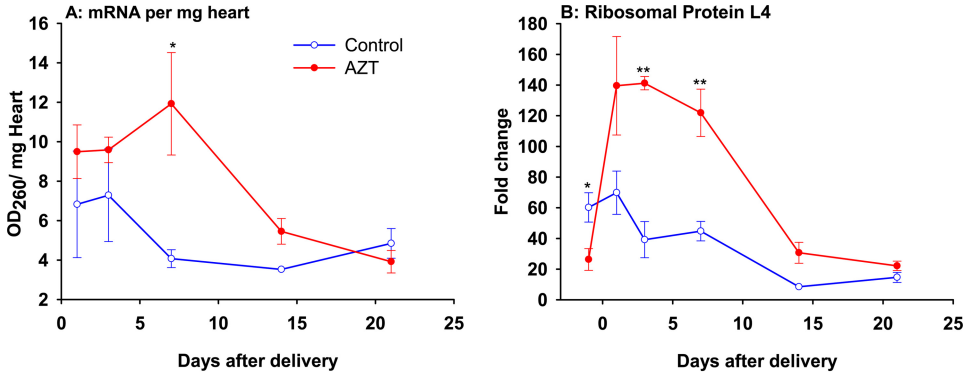

FIG 1 Effects of AZT treatment on mRNA levels and RPL4 gene. mRNA and cDNA were obtained from hearts of age-matched controls and AZT-treated embryonic (day −1) and neonatal (postgestation days 1, 3, 7, 14, and 21) pups as described in Materials and Methods. (A) mRNA was quantified and expressed as OD_{260} value per milligram of heart tissue. (B) Effect of AZT on housekeeping (RpL4) gene mRNA expression level. Results are expressed as described in Materials and Methods as fold changes from cycle time of adult control Dguok for both treated and aged-matched control samples. All data points represent means and SEM from three independent determinations from three different rat heart isolates. Student's *t* test was performed to analyze the significance between the control and the treated groups at each time point. *, $P < 0.05$; **, $P < 0.01$.

verted to dNTP concentration via a standard curve generated using known amounts of dNTP in the assay and normalized to milligrams of heart tissue used. Final dNTP levels were expressed as picomoles/milligram tissue.

Reverse transcription. For reverse transcription, 800 ng mRNA (OD_{260}; NanoDrop) was used for each tissue time point. As shown in Fig. 1A, AZT increased the amount of mRNA isolated per milligram of tissue during the first week of treatment. Thus, less tissue was used for reverse transcription in the AZT-treated hearts than in control hearts at these points. The isolated mRNA was reverse transcribed to cDNA using the High Capacity RNA-to-cDNA kit from Applied Biosystems and the enzyme reverse transcriptase.

RT-PCR. Thirty nanograms (OD_{260}; NanoDrop) of cDNA obtained from the reverse transcription was analyzed by real-time PCR (RT-PCR) using the Power SYBR green PCR master mix (Applied Biosystems). Primers designed by Oligo 7 software (Molecular Biology Insights, Inc.) and synthesized by Integrated DNA Technologies Inc. (Coralville, IA) (Table 1) were used to quantitate mRNA expression levels of target genes. The selected mRNA expression levels were measured from embryonic (day −1) and neonatal samples on the 1st, 3rd, 7th, 14th, and 21st days postgestation. As tissues convert from hyperplastic to hypertrophic growth, the expression of many mRNAs, including mRNA of housekeeping proteins, may change, as was noted for ribosomal protein L4 (RpL4) (Fig. 1B), which decreased 5-fold per aliquot of cDNA from birth to 3 weeks of growth in the age-matched controls. AZT significantly increased the level of mRNA for RpL4 2- to 4-fold during the first week of growth. As a result, it was not possible to compare the mRNA levels of our target enzymes to that of a housekeeping protein. Instead, as noted above, we were careful to take exactly the same amount of mRNA (800 ng, OD_{260}) from each sample and to take exactly the same amount of reverse-transcribed product (30 ng, OD_{260}) from each sample for RT-PCR. In addition, we prepared a large mRNA sample from an adult rat heart control. A sample of this was used in each RT-PCR experiment to generate a standard graph in which the mRNA expression level of deoxyguanosine kinase (Dguok) was measured within a 3-fold range. The cycle time at the midpoint for adult rat heart Dguok from experiment to experiment ($n = 8$)

TABLE 1 Primers used for real-time PCR[a]

Target gene (abbreviation; nucleotide positions)	Primer sequence	
	Forward (5′–3′)	Reverse (5′–3′)
Ribonucleotide reductase M1 (Rrm1; 518–655)	ATAAATCCACACAACGGCAGA	ATAAATCCACACAACGGCAGA
Ribonucleotide reductase M2 (Rrm2; 228–309)	AAGTAAAGCACCCACTAAGCC	AAGTAAAGCACCCACTAAGCC
Ribonucleotide reductase, p53 inducible (Rrm2b; 243–453)	TCCTCAGAAAGAGTTCCCGAC	CCTCTTGACTAAAACGCTCCA
Thymidylate synthase (Tyms; 1326–1490)	CCCTAAGCCTTCATTAGCTC	ATAAATCAAAAGGCACTACACC
Thymidine kinase 1 (Tk1; 1143–1366)	CTCGGGTCTCACATTCAGC	GGCACAAGAAACATACTCCCT
Deoxycytidine kinase (Dck; 555–867)	ATTTATCTTCGAGCTACTCCA	CAGTAATATCAAGCTATGGCAA
Mitochondrial transcription termination factor (MTERF; 173–239)	TTTAACCCTAAAGGCTTCGT	TTTCTTGATGCCATTATCACA
Mitochondrial transcriptional factor B2 (TFB2 M; 7713–7889)	TGATCTGTACTCCTGCGAAT	ATGCACACTAAATGACGACCA
Ribosomal protein L4 (Rpl4; 965–1138)	CACGCAAGAAGATTCATCGC	AACAATCTTCTCCGATTTGGC
Thymidylate kinase (Dtymk; 359–574)	AGATCAACAGAAATCGGCAAG	AGATCAACAGAAATCGGCAAG
Thymidine phosphorylase (Tymp; 1816–1945)	GACTTCAGCCTACTGGACCAC	GACTTCAGCCTACTGGACCAC
SAM domain and HD domain-containing protein 1 (SAMHD; 931–1014)	GGCACAAGAAACATACTCCCT	CCCTTATACGGCCACAAGCAG
Dihydrofolate reductase (Dhfr; 141–374)	CAAGAACGGAGACTTACCCTG	CAAGAACGGAGACTTACCCTG
Thymidine kinase 2 (Tk2; 428–677)	AAAGGTCAATTTACAGCGCAAG	GTGACCAGCCACTCCTCGT
Deoxyguanosine kinase (Dguok; 546–690)	TCGAATGGCACATCTATCAGG	TCGAATGGCACATCTATCAGG
Mitochondrial transcriptional factor B1 (TFB1 M; 721–805)	TACAGCCCAAGATCAAGCA	TACAGCCCAAGATCAAGCA
Cytochrome *c* oxidase 1 (COX1)	TAATTCGAGCTGAACTAGGAC	TACAAGTCAGTTCCCGAAGC

[a] Primers were designed using Oligo 7 software (Molecular Biology Insights, Inc.) and synthesized by Integrated DNA Technologies Inc. (Coralville, IA) (see Materials and Methods).

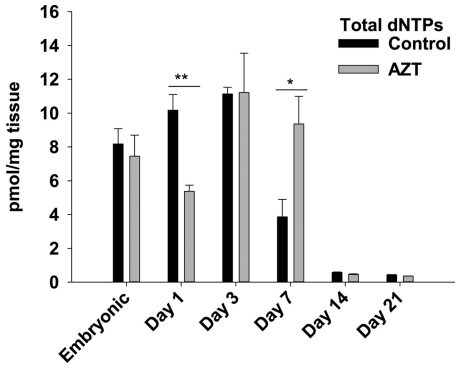

FIG 2 Total dNTP pool in embryonic and neonatal pup heart tissue. dNTP pools from hearts of age-matched controls and AZT-treated (100 mg/kg body weight) embryonic (day −1) and neonatal (postgestation days 1, 3, 7, 14, and 21) pups were quantified using a modified template-driven DNA polymerase assay described in Materials and Methods. The sums of all four dNTPs from each heart tissue were expressed as picomoles per milligram of tissue. All data points represent means and SEM from three independent determinations from three different rat heart isolates. Student's *t* test was performed to analyze the significance between the control and the treated groups at each time point. *, $P < 0.05$; **, $P < 0.01$.

was 24.1 ± 0.4 (average ± standard error of the mean [SEM]) cycle times. The midpoint cycle time observed for each individual experiment for the adult rat heart control Dguok mRNA was set as a relative value of 1, and all cycle time data for mRNAs of our target enzymes in both the treated and age-matched controls were expressed as fold changes from this value. Dguok was chosen because it represented a reasonable midpoint cycle time for all the data. It was also noted that while the levels of Dguok in the neonatal samples were a bit lower than in the adults (0.6 to 0.8), they were close and changed relatively little during the neonatal time course of this study.

Mitochondrial DNA copy number. In rat tissues, 25 to 50 mg of frozen sample was used for total DNA isolation as described previously. Quantitative real-time PCR was performed using 40 ng DNA (OD_{260}; NanoDrop) as the starting material with the designed primers (Oligo 7 software) for the mitochondrial gene cytochrome *c* oxidase subunit 1(COX1) and the nuclear gene encoding ribosomal protein L4 (RpL4). The level of mitochondrial DNA copy number was determined by subtracting the mitochondrial gene (COX1 gene) cycle time from that of the nuclear gene (RpL4 gene) and expressed as mtDNA copy number per diploid nuclear genome.

Data analysis. Data presented in the figures represent the means and SEM from at least three independent determinations from three individual rat heart tissue isolates. Significant differences between results for age-matched control and AZT-treated animals were computed via Student's *t* test at each time point.

RESULTS

Time course on dNTP levels and composition in control embryonic and neonatal rat heart. The total dNTP pool size was determined using rat hearts collected from the last day of pregnancy (day −1) and on postgestation days 1, 3, 7, 14, and 21. As shown in Fig. 2 (control), the sum of the four dNTPs (total dNTPs) were highest in embryonic (day −1) and postgestation day 1 and day 3 rat hearts (8.2, 10.2, and 11.1 pmol/mg tissue, respectively). The total dNTP pool size dramatically shrank, by more than 50% by day 7 (3.9 pmol/mg tissue), and it was further reduced to just 6% of the highest level observed by day 14 (0.6 pmol/mg tissue) and maintained a low level through day 21 (0.4 pmol/mg tissue). dNTP pool composition (Fig. 3, control) altered dramatically throughout the neonatal growth period, with dATP and TTP being the predominate triphosphates on days −1, 1, 3, 7, and 14. However, as the total dNTP pools shrunk significantly on day 14, the relative ratio of dGTP of the total dNTP pools increased substantially through day 21 and adult (Fig. 3, control). dCTP pools remained at a relatively consistent proportion throughout all time periods.

Effect of AZT treatment on time course dNTP levels and composition in embryonic and neonatal rat heart. The total dNTP pool size on day 1 from AZT-treated tissues was significantly lower than that of age-matched controls (Fig. 2). On day 3, the treated pools were restored to age-matched control levels but then became significantly higher than the age-matched control on day 7. By day 14, the treated pools decreased to age-matched control levels and remained at a low level through day 21. The effect of AZT on dNTP pool asymmetries is shown in Fig. 3. TTP as a percentage of the total pool was reduced compared to age-matched control on day 1 but rebounded to age-matched control proportions at subsequent time points. Surprisingly, the major asymmetry observed in the AZT-treated tissue was a profound reduction in the proportion of dGTP compared to age-matched control tissue, which did not rebound until day 7 (Fig. 3). The decrease in dGTP and TTP in the 1-day and 3-day AZT-treated tissues resulted in an increase in the proportion of the pool that is dATP compared to age-matched controls.

To obtain a clearer understanding of the effects of AZT on dNTP pools, the absolute level of each pool (in picomoles per milligram of tissue) is shown in Fig. 4. TTP pools were reduced by 67% on day 1, but the level rebounded by day 3 and overshot by day 7 in comparison to the levels of age-matched controls (Fig. 4A). The profound drop in the proportion of dGTP noted above and illustrated in Fig. 3 was confirmed with an absolute drop of >90% on day 1 and day 3 compared to age-matched controls (Fig. 4C). As described above, these results normalized to those of age-matched controls by day 7 as the age-matched control level of dGTP dramatically fell. The dATP pool responded to AZT treatment with a 41.5% increase on day 3 increasing to a 58.9% increase on day 7 compared to age-matched controls. As with the others, these differences normalized on day 14 and day 21 as the dATP levels fell (Fig. 4D). There were no significant differences in the dCTP pools in either absolute or proportional amounts upon AZT treatment (Fig. 4B).

Time course of mRNA expression of the enzymes of dNTP synthesis and salvage in control embryonic and neonatal rat heart. As tissues convert from hyperplastic to hypertrophic growth, the size of the total dNTP pool was shown to dramatically decrease. We hypothesized that the expression of the enzymes of dNTP synthesis and salvage would also decline during this growth transition. As shown in Fig. 5, many of the enzymes under control conditions showed a dramatic decrease in mRNA expression, including ribonucleotide reductases 1 (Fig. 5A) and 2 (Fig. 5B), thymidylate synthase (Fig. 5D), thymidine kinase 1 (Fig. 5F), and to a lesser extent thymidine kinase 2 (Fig. 5H) and SAMHD1 (a phosphohydrolase that cleaves dNTPs to nucleoside and triphosphate) (Fig. 5L). Conversely, the levels of ribonucleotide reductase 2B (p53 inducible) (Fig. 5C), dihydrofolate reductase (Fig. 5E), deoxycytidine kinase (Fig. 5G), deoxyguanosine kinase (Fig. 5I), deoxythymidylate kinase (Fig. 5J), and thymidine phosphorylase (Fig. 5K) were relatively unchanged during the control time course.

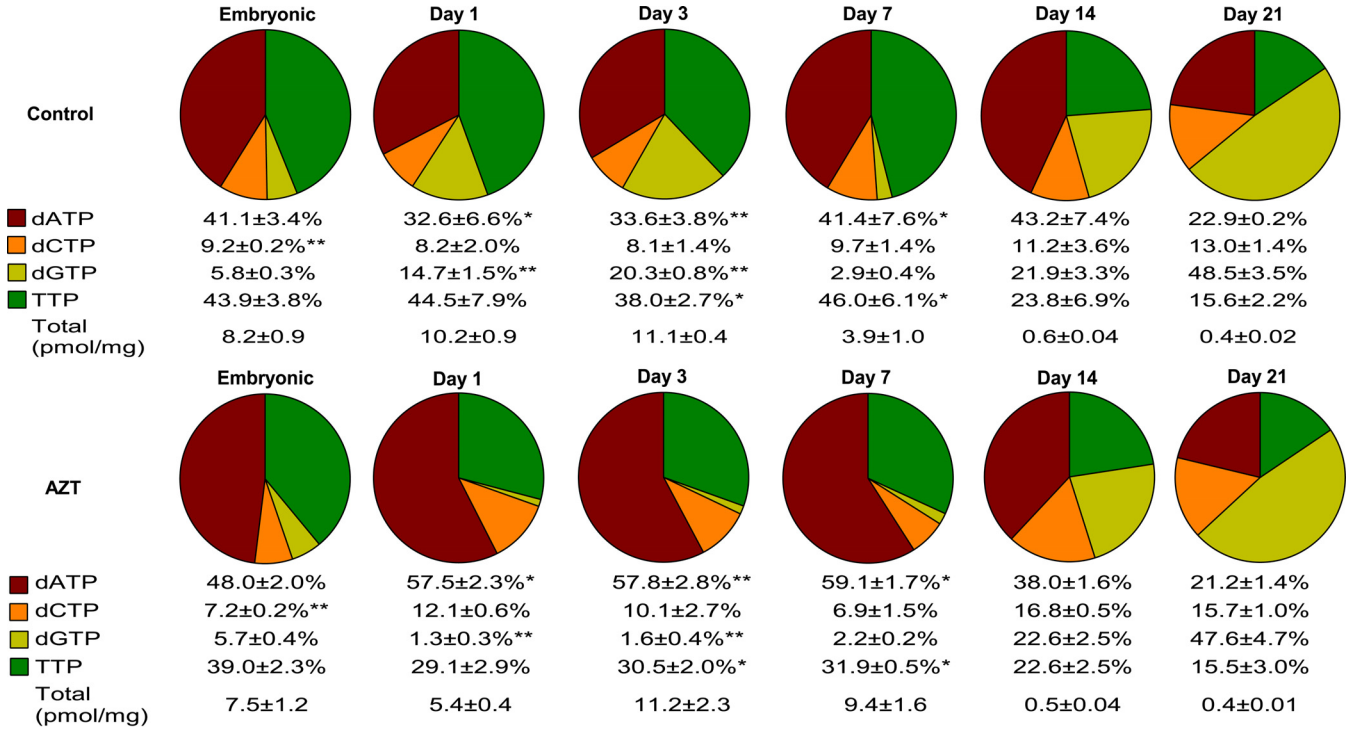

FIG 3 Effects of AZT treatment on total dNTP pool composition and asymmetry in embryonic and neonatal pup heart tissue. dNTP pools from age-matched controls and AZT-treated rat heart tissues were prepared and quantified as described in Materials and Methods and the legend to Fig. 2. The composition of each dNTP is expressed as a percentage of the total dNTP of the heart at each time point. Totals represent the absolute amount of total dNTP expressed as picomoles per milligram of tissue. All data points represent means and SEM from three independent determinations from three different rat heart isolates. Student's *t* test was performed to analyze the significance of the percent composition of each dNTP between the control and the treated groups at each time point. *, $P < 0.05$; **, $P < 0.01$.

Effects of AZT treatment on time course of mRNA expression of the enzymes of dNTP synthesis and salvage in embryonic and neonatal rat heart. AZT is a nucleoside analogue known to inhibit thymidine kinases and shown above to disrupt the composition of dNTP pools. Thus, we wanted to determine whether AZT directly or indirectly altered the gene expression of enzymes involved in the synthesis and salvage of dNTPs. In the AZT-treated heart tissue, expression of enzyme mRNAs of *de novo* dNTP synthesis and salvage pathways were significantly higher than in age-matched control heart tissue in the early days following birth. AZT induced large increases in these mRNA levels peaking at day 1 or day 3 at levels 3- to 6-fold higher than equivalent age-matched control values. Following this peak, as shown in Fig. 5, these mRNA levels dramatically fell to age-matched controls by day 14 and day 21 with the exceptions that ribonucleotide reductase 2B (Fig. 5C), thymidine kinase 2 (Fig. 5H), and deoxyguanosine kinase (Fig. 5I) levels remained significantly higher than the age-matched control levels at day 14 and day 21.

Time course of mRNA expression of mitochondrial transcription/termination factors. As shown in Fig. 5, only modest changes were noted in the time course of controls for mRNAs of enzymes associated with mitochondrial biogenesis, including mitochondrial transcription termination factor (Fig. 5M) and mitochondrial transcription factors B1 (Fig. 5N) and B2 (Fig. 5O).

Effect of AZT treatment on the time course of mRNA expression of mitochondrial transcription/termination factors. Recognizing that AZT treatment is associated with mitochondrial dysfunction, we investigated the effect of AZT treatment on mRNAs of proteins associated with mitochondrial biogenesis. AZT treatment induced a large increase in mRNA expression for the transcription and termination factors, peaking at days 1 and 3 at levels 3- to 9-fold above age-matched control values (Fig. 5M to O). The levels of these AZT-treated mRNAs steeply decreased to approach those of the age-matched control values by day 21 with the exception of mitochondrial transcription factor B1 (Fig. 5N), which remained elevated.

Time course of mitochondrial DNA copy numbers in control embryonic and neonatal rat heart. In Fig. 6, DNA copy number was examined for the mitochondrial gene encoding cytochrome *c* oxidase subunit 1 (COX1) and compared to the copy number for the nuclear gene encoding ribosomal protein L4 (RpL4). The ratio of mitochondrial to nuclear DNA copy number increased 4-fold from the embryonic time point day −1 through 21 days after birth.

Effects of AZT treatment on the time course of mitochondrial DNA copy numbers in embryonic and neonatal rat heart. AZT treatment resulted in a nonsignificant increase in the mitochondrial-to-nuclear DNA ratio at day −1, day 1, and day 3 (Fig. 6). However, as all three time points show the same trend, this modest amplification may be real. On days 7 and 14, a significant reduction in copy numbers was seen in the AZT-treated heart tissue. Copy numbers in the AZT-treated tissues returned to age-matched control levels, and no significant difference was seen on day 21.

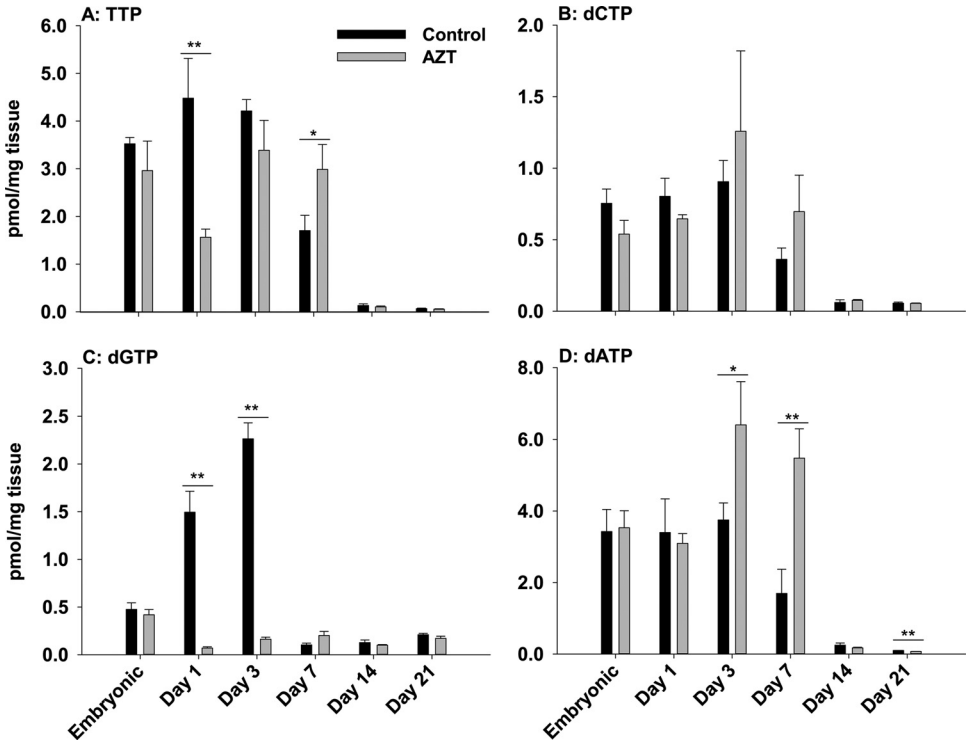

FIG 4 Effects of AZT treatment on individual dNTP concentrations in embryonic and neonatal pup heart tissue. dNTPs from age-matched controls and AZT-treated rat heart tissues were extracted and quantified as described in Materials and Methods and the legend to Fig. 2. The concentration of each dNTP is expressed in picomoles per milligram of tissue. (A) TTP; (B) dCTP; (C) dGTP; (D) dATP. All data points represent means and SEM from three independent determinations from three different rat heart isolates. Student's *t* test was performed to analyze the significance between the control and the treated groups at each time point. *, $P < 0.05$; **, $P < 0.01$.

DISCUSSION

Previous studies in development have shown that tissues such as the heart undergo extensive cell division (hyperplasia) during the first week or so of growth after birth. Following this period, while growth continues, cell division stops and growth is due to hypertrophy (24, 25). In cell culture, it is well known that the size of the dNTP pools and the expression of enzymes responsible for their synthesis and salvage are much higher in dividing cells than in quiescent cells (31). We have shown in this study that a similar change takes place *in vivo*, in which dNTP pools and the enzymes responsible for their synthesis and salvage decreased dramatically as heart tissue moved from hyperplasia to hypertrophy (Fig. 2 and 5). We hypothesize that the effect of a nucleoside analogue such as AZT would be significantly different in early hyperplastic growth as opposed to hypertrophic growth in adult animals.

AZT has been extensively used to prevent the transmission of HIV infection from mothers to offspring. In the past, AZT was used to treat the mothers prior to and during birth as well as the neonates after birth (1–3, 5, 17, 18). While more recently developed NRTIs that are known to be less toxic are now being used to treat the mothers before and during birth, AZT is still recommended to treat neonates (4). Despite the past routine usage of AZT in mothers and the continuing use in neonates, the metabolism and mechanism(s) of toxicity of AZT or other analogues in the NRTI group have been poorly studied in neonates. Past studies have shown mitochondrial DNA depletion involving adult rat or mouse populations when treated with oral AZT (15, 16, 32). Our data show for the first time that AZT dramatically altered the size and composition of the dNTP pools during early neonatal growth (Fig. 2 to 4). In the first day of treatment after birth, AZT decreased TTP levels by 67% and dGTP by 95%. While TTP levels recover on day 3 and overshoot on day 7, dGTP levels remained low compared to those of age-matched controls until day 7. Conversely, AZT treatment led to a 2- to 3-fold increase in dATP compared to age-matched controls on day 3 and day 7. These effects taken together clearly indicate that the relative composition of the dNTP pools is severely altered during the first week of AZT treatment. All of the dNTP pools appeared to normalize on days 14 and 21.

It is known that thymidine kinase 1 is expressed during S phase in hyperplastic cells, and it appears to be induced in this study by AZT treatment and is reported to be primarily responsible for AZT phosphorylation (33). From this, we propose that much higher levels of AZT-TP are present in neonatal hyperplastic heart tissue than are likely to be observed in adult tissue. Thus, AZT-TP may have effects in early neonatal heart that would not be observed in the adult. While these effects are poorly understood, one possibility is that AZT-TP may affect ribonucleotide reductase in such a way as to reduce the reduction of GDP and enhance the reduction of ADP, leading to the observed decrease in dGTP.

Dramatic increases in the levels of many mRNAs of the enzymes of the *de novo* dNTP synthesis and salvage pathways were associated with the changes noted in the dNTP pools in the first week. These increases peaked at days 1 and 3 and returned to age-matched control values by days 14 and 21 (Fig. 5). The time frame of changes in mRNA levels correlated with the time frame changes in the dNTP pools. However, it was not possible to deter-

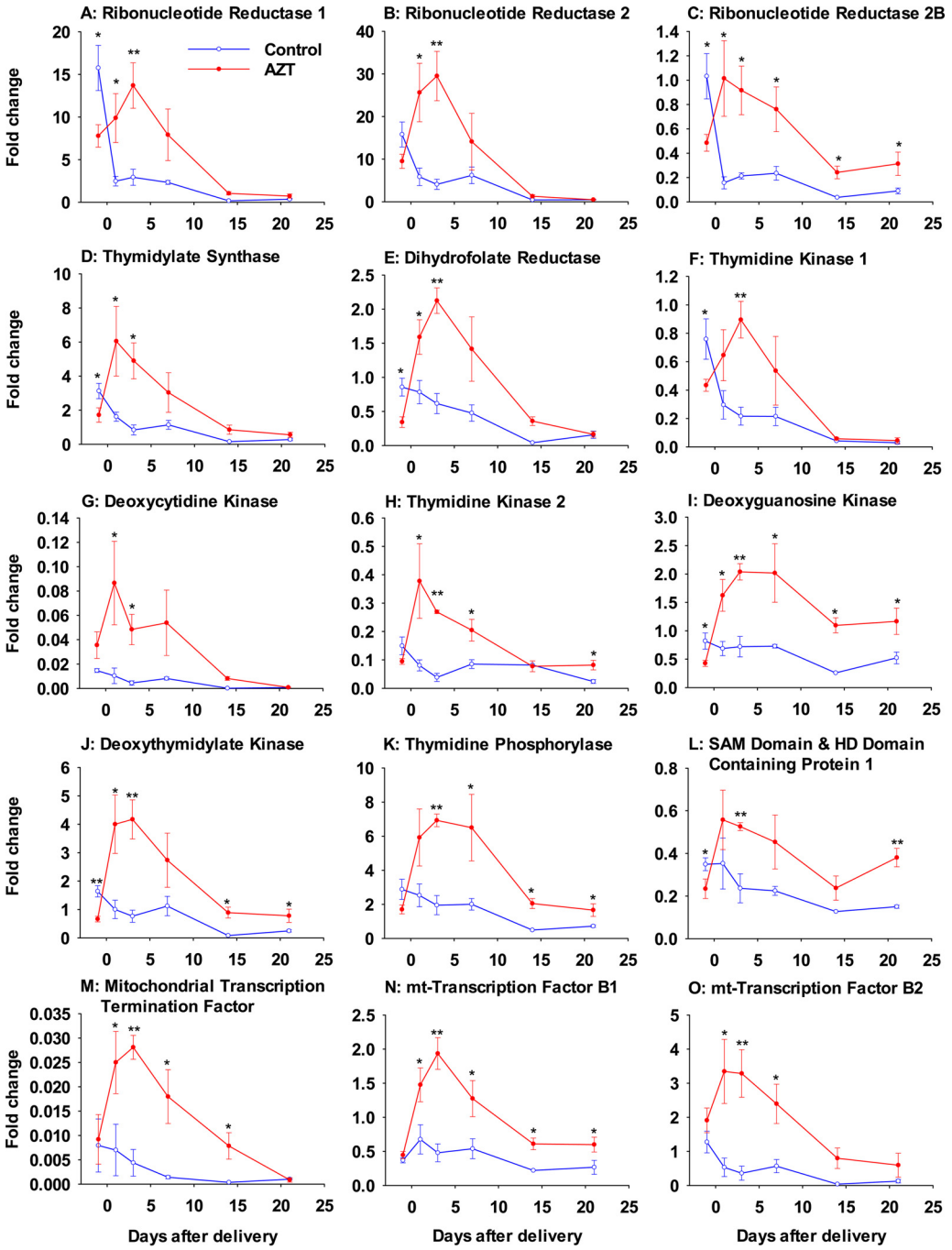

FIG 5 mRNA expression in embryonic and neonatal rat heart tissue. Thirty nanograms of heart cDNA obtained from age-matched controls and AZT-treated embryonic (day −1) and neonatal (postgestation days 1, 3, 7, 14, and 21) pups was used in real-time PCR as described in Materials and Methods. mRNA expression levels were expressed as fold changes as described in the legend to Fig. 1. Primer sequences used in this study are shown in Table 1. All data points represent means and SEM from three independent determinations from three different rat heart isolates. Student's *t* test was performed to analyze the significance between the control and the treated groups at each time point. *, $P < 0.05$; **, $P < 0.01$.

mine if these observations were separate effects of AZT treatment or were causally related to each other. We have previously shown that AZT inhibits thymidine phosphorylation and reduces TTP levels (22). As expected, TTP levels fell on day 1 in response to AZT treatment but recovered and overshot by day 7. This may be related to much higher levels of thymidine kinase 1 and thymidine kinase 2 mRNA associated with AZT treatment. However, there was no rebound in the AZT-induced reduction of dGTP, even though the mRNA for deoxyguanosine kinase was similarly increased. Alternatively, AZT or phosphorylated AZT may have had direct effects on mRNA levels. Clearly, AZT treatment had a significant impact in increasing overall mRNA levels per milligram of tissue (Fig. 1A) during the first week of hyperplastic growth. It is not clear if the effect on mRNA levels is at the level of transcription

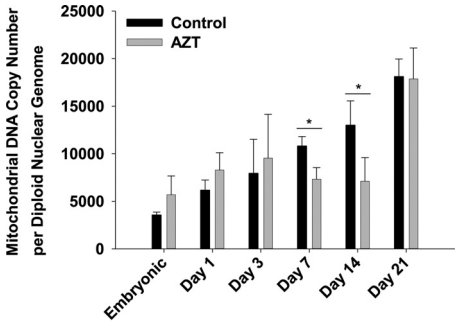

FIG 6 Mitochondrial DNA copy numbers in embryonic and neonatal rat heart tissue. Genomic and mitochondrial DNAs from age-matched controls and AZT-treated rat hearts were isolated as described in Materials and Methods. Expression of mitochondrial DNA copy number was quantified by subtracting the mitochondrial gene (COX1 gene) cycle time from that of the nuclear gene (RpL4 gene) and expressed as mtDNA copy number per diploid nuclear genome. All data represent means and SEM from three independent determinations from three different rat heart isolates. Student's *t* test was performed to analyze the significance between the control and the treated groups at each time point. *, $P < 0.05$.

or at the level of mRNA stability. It is important to note that increased mRNA levels do not necessarily reflect increased enzyme levels; nor do increased enzyme levels necessarily reflect increased levels of dNTPs, as other factors such as substrate availability and dNTP utilization may play important roles. Interestingly, the effect of AZT treatment on mRNA is lost by postgestational days 14 and 21, when heart tissue has converted to hypertrophic growth. If AZT or phosphorylated AZT is directly affecting gene expression or mRNA stability, it can do so only in the hyperplastic state.

Concomitant with the effects of AZT on the mRNAs of the *de novo* dNTP synthesis and salvage pathways, AZT treatment dramatically increased the levels of mRNAs of enzymes associated with mitochondrial biogenesis. This increase may reflect mitochondrial dysfunction and an attempt by the tissue to compensate by increasing the number of mitochondria, perhaps accounting for the small increase in the mitochondrial-to-nuclear DNA ratio observed during the first 3 days of growth compared to age-matched controls (Fig. 6). However, the potential amplification in mtDNA gave way to significant reductions observed on day 7 and day 14, suggesting instability in mtDNA, perhaps related to errors in mtDNA caused by the noted altered composition of the dNTP pools. As the composition of the dNTP pools normalized on days 14 and 21, mtDNA levels returned to normal. Depletion in mtDNA copy numbers has been shown to compromise oxidative phosphorylation capacity in mitochondria, causing respiratory chain dysfunction in tissues (34).

The compiled data from this study are direct evidence of the AZT treatment-induced changes in mRNA expression of nucleoside metabolism, alteration in dNTP pool composition, and depletion of mitochondrial DNA. While these data were obtained using a suprapharmacological dose modeled on earlier rodent studies (15, 28), they nevertheless provide proof of principle of potential toxicity to human neonates. Clearly, further *in vivo* studies must be conducted to establish a dose-response analysis. Short-term (7–14) and long-term (15–18) problems associated with AZT treatment in neonatal animal models and humans have been noted by others and are described in the introduction. It has been previously shown that alterations in dNTP composition are associated with mutagenesis in nuclear and mitochondrial DNA (23, 26) and may account for the long-term problems (15–18) and reports of AZT-induced carcinogenicity (15). While not a goal of this study, an examination of the implications of the AZT-induced cellular changes noted here on both short-term and long-term health of the animals is planned. Lastly, it is unknown if the results presented here are specific to AZT or are more broadly associated with this class of compounds. Thus, studies with less toxic analogs, such as the emtricitabine/tenofovir (Truvada) combination, must be performed and are planned.

ACKNOWLEDGMENT

This research was funded by National Institutes of Health R01/HL096480.

REFERENCES

1. **Connor EM, Sperling RS, Gelber R, Kiselev P, Scott G, O'Sullivan MJ, VanDyke R, Bey M, Shearer W, Jacobson RL.** 1994. Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group. N Engl J Med **331:**1173–1180.
2. **Cooper ER, Charurat M, Mofenson L, Hanson IC, Pitt J, Diaz C, Hayani K, Handelsman E, Smeriglio V, Hoff R, Blattner W.** 2002. Combination antiretroviral strategies for the treatment of pregnant HIV-1-infected women and prevention of perinatal HIV-1 transmission. J Acquir Immune Defic Syndr **29:**484–494. http://dx.doi.org/10.1097/00042560-200204150-00009.
3. **WHO.** 2010. Antiretroviral therapy for HIV infection in infants and children: towards universal access. Recommendations for a public health approach. World Health Organization, Geneva, Switzerland.
4. **WHO.** 2013. Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection: recommendations for a public health approach, June 2013. World Health Organization, Geneva, Switzerland.
5. **Persaud D, Gay H, Ziemniak C, Chen YH, Piatak M, Chun TW, Strain M, Richman D, Luzuriaga K.** 2013. Absence of detectable HIV-1 viremia after treatment cessation in an infant. N Engl J Med **369:**1828–1835. http://dx.doi.org/10.1056/NEJMoa1302976.
6. **Richman DD, Fischl MA, Grieco MH, Gottlieb MS, Volberding PA, Laskin OL, Leedom JM, Groopman JE, Mildvan D, Hirsch MS.** 1987. The toxicity of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial. N Engl J Med **317:**192–197.
7. **Fernández Ibieta M, Ramos Amador JT, González Tomé MI, Guillén Martín S, Bellón Cano JM, Navarro Gómez M, de José MI, Beceiro J, Iglesias E, Rubio B, Relaño Garrido P, Santos MJ, Martínez Guardia N, Roa MA, Regidor J.** 2008. Anaemia and neutropenia in a cohort of non-infected children of HIV-positive mothers. An Pediatr (Barc) **69:**533–543. (In Spanish.) http://dx.doi.org/10.1016/S1695-4033(08)75236-6.
8. **Lipshultz SE, Shearer WT, Thompson B, Rich KC, Cheng I, Orav EJ, Kumar S, Pignatelli RH, Bezold LI, LaRussa P, Starc TJ, Glickstein JS, O'Brien S, Cooper ER, Wilkinson JD, Miller TL, Colan SD.** 2011. Cardiac effects of antiretroviral therapy in HIV-negative infants born to HIV-positive mothers: NHLBI CHAART-1 (National Heart, Lung, and Blood Institute Cardiovascular Status of HAART Therapy in HIV-Exposed Infants and Children cohort study). J Am Coll Cardiol **57:**76–85. http://dx.doi.org/10.1016/j.jacc.2010.08.620.
9. **Lewis W, Grupp IL, Grupp G, Hoit B, Morris R, Samarel AM, Bruggeman L, Klotman P.** 2000. Cardiac dysfunction occurs in the HIV-1 transgenic mouse treated with zidovudine. Lab Invest **80:**187–197. http://dx.doi.org/10.1038/labinvest.3780022.
10. **Zuena AR, Giuli C, Venerosi Pesciolini A, Tramutola A, Ajmone-Cat MA, Cinque C, Alemà GS, Giovine A, Peluso G, Minghetti L, Nicolai R, Calamandrei G, Casolini P.** 2013. Transplacental exposure to AZT induces adverse neurochemical and behavioral effects in a mouse model: protection by L-acetylcarnitine. PLoS One **8:**e55753. http://dx.doi.org/10.1371/journal.pone.0055753.
11. **Desai VG, Lee T, Moland CL, Branham WS, Von Tungeln LS, Beland**

FA, Fuscoe JC. 2009. Effect of short-term exposure to zidovudine (AZT) on the expression of mitochondria-related genes in skeletal muscle of neonatal mice. Mitochondrion **9:**9–16. http://dx.doi.org/10.1016/j.mito.2008.09.002.

12. **Liu Y, Nguyen P, Baris TZ, Poirier MC.** 2012. Molecular analysis of mitochondrial compromise in rodent cardiomyocytes exposed long term to nucleoside reverse transcriptase inhibitors (NRTIs). Cardiovasc Toxicol **12:**123–134. http://dx.doi.org/10.1007/s12012-011-9148-5.
13. **Divi RL, Leonard SL, Walker BL, Kuo MM, Shockley ME, St Claire MC, Nagashima K, Harbaugh SW, Harbaugh JW, Poirier MC.** 2007. Erythrocebus patas monkey offspring exposed perinatally to NRTIs sustain skeletal muscle mitochondrial compromise at birth and at 1 year of age. Toxicol Sci **99:**203–213. http://dx.doi.org/10.1093/toxsci/kfm143.
14. **Gerschenson M, Nguyen V, Ewings EL, Ceresa A, Shaw JA, St Claire MC, Nagashima K, Harbaugh SW, Harbaugh JW, Olivero OA, Divi RL, Albert PS, Poirier MC.** 2004. Mitochondrial toxicity in fetal Erythrocebus patas monkeys exposed transplacentally to zidovudine plus lamivudine. AIDS Res Hum Retroviruses **20:**91–100. http://dx.doi.org/10.1089/088922204322749530.
15. **Walker DM, Malarkey DE, Seilkop SK, Ruecker FA, Funk KA, Wolfe MJ, Treanor CP, Foley JF, Hahn FF, Hardisty JF, Walker VE.** 2007. Transplacental carcinogenicity of 3′-azido-3′-deoxythymidine in B6C3F1 mice and F344 rats. Environ Mol Mutagen **48:**283–298. http://dx.doi.org/10.1002/em.20297.
16. **Walker DM, Poirier MC, Campen MJ, Cook DL, Divi RL, Nagashima K, Lund AK, Cossey PY, Hahn FF, Walker VE.** 2004. Persistence of mitochondrial toxicity in hearts of female B6C3F1 mice exposed in utero to 3′-azido-3′-deoxythymidine. Cardiovasc Toxicol **4:**133–153. http://dx.doi.org/10.1385/CT:4:2:133.
17. **Barret B, Tardieu M, Rustin P, Lacroix C, Chabrol B, Desguerre I, Dollfus C, Mayaux MJ, Blanche S, French Perinatal Cohort Study Group.** 2003. Persistent mitochondrial dysfunction in HIV-1-exposed but uninfected infants: clinical screening in a large prospective cohort. AIDS **17:**1769–1785. http://dx.doi.org/10.1097/00002030-200308150-00006.
18. **Tardieu M, Brunelle F, Raybaud C, Ball W, Barret B, Pautard B, Lachassine E, Mayaux MJ, Blanche S.** 2005. Cerebral MR imaging in uninfected children born to HIV-seropositive mothers and perinatally exposed to zidovudine. AJNR Am J Neuroradiol **26:**695–701.
19. **Lewis W, Simpson JF, Meyer RR.** 1994. Cardiac mitochondrial DNA polymerase-gamma is inhibited competitively and noncompetitively by phosphorylated zidovudine. Circ Res **74:**344–348. http://dx.doi.org/10.1161/01.RES.74.2.344.
20. **Lynx MD, McKee EE.** 2006. 3′-Azido-3′-deoxythymidine (AZT) is a competitive inhibitor of thymidine phosphorylation in isolated rat heart and liver mitochondria. Biochem Pharmacol **72:**239–243. http://dx.doi.org/10.1016/j.bcp.2006.04.004.
21. **McCann KA, Williams DW, McKee EE.** 2012. Metabolism of deoxypyrimidines and deoxypyrimidine antiviral analogs in isolated brain mitochondria. J Neurochem **122:**126–137. http://dx.doi.org/10.1111/j.1471-4159.2012.07765.x.
22. **Morris GW, Iams TA, Slepchenko KG, McKee EE.** 2009. Origin of pyrimidine deoxyribonucleotide pools in perfused rat heart: implications for 3′-azido-3′-deoxythymidine-dependent cardiotoxicity. Biochem J **422:**513–520. http://dx.doi.org/10.1042/BJ20082427.
23. **Song S, Pursell ZF, Copeland WC, Longley MJ, Kunkel TA, Mathews CK.** 2005. DNA precursor asymmetries in mammalian tissue mitochondria and possible contribution to mutagenesis through reduced replication fidelity. Proc Natl Acad Sci U S A **102:**4990–4995. http://dx.doi.org/10.1073/pnas.0500253102.
24. **Li F, Wang X, Gerdes AM.** 1997. Formation of binucleated cardiac myocytes in rat heart. II. Cytoskeletal organisation. J Mol Cell Cardiol **29:**1553–1565. http://dx.doi.org/10.1006/jmcc.1997.0403.
25. **Li F, Wang X, Capasso JM, Gerdes AM.** 1996. Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. J Mol Cell Cardiol **28:**1737–1746. http://dx.doi.org/10.1006/jmcc.1996.0163.
26. **Ashley N, Adams S, Slama A, Zeviani M, Suomalainen A, Andreu AL, Naviaux RK, Poulton J.** 2007. Defects in maintenance of mitochondrial DNA are associated with intramitochondrial nucleotide imbalances. Hum Mol Genet **16:**1400–1411. http://dx.doi.org/10.1093/hmg/ddm090.
27. **Kunz BA, Kohalmi SE.** 1991. Modulation of mutagenesis by deoxyribonucleotide levels. Annu Rev Genet **25:**339–359. http://dx.doi.org/10.1146/annurev.ge.25.120191.002011.
28. **Desai VG, Lee T, Delongchamp RR, Leakey JE, Lewis SM, Lee F, Moland CL, Branham WS, Fuscoe JC.** 2008. Nucleoside reverse transcriptase inhibitors (NRTIs)-induced expression profile of mitochondria-related genes in the mouse liver. Mitochondrion **8:**181–195. http://dx.doi.org/10.1016/j.mito.2008.01.002.
29. **Sherman PA, Fyfe JA.** 1989. Enzymatic assay for deoxyribonucleoside triphosphates using synthetic oligonucleotides as template primers. Anal Biochem **180:**222–226. http://dx.doi.org/10.1016/0003-2697(89)90420-X.
30. **Mathews CK, Wheeler LJ.** 2009. Measuring DNA precursor pools in mitochondria. Methods Mol Biol **554:**371–381. http://dx.doi.org/10.1007/978-1-59745-521-3_22.
31. **Gandhi VV, Samuels DC.** 2011. A review comparing deoxyribonucleoside triphosphate (dNTP) concentrations in the mitochondrial and cytoplasmic compartments of normal and transformed cells. Nucleosides Nucleotides Nucleic Acids **30:**317–339. http://dx.doi.org/10.1080/15257770.2011.586955.
32. **Chan SS, Santos JH, Meyer JN, Mandavilli BS, Cook DL, Jr, McCash CL, Kissling GE, Nyska A, Foley JF, van Houten B, Copeland WC, Walker VE, Witt KL, Bishop JB.** 2007. Mitochondrial toxicity in hearts of CD-1 mice following perinatal exposure to AZT, 3TC, or AZT/3TC in combination. Environ Mol Mutagen **48:**190–200. http://dx.doi.org/10.1002/em.20191.
33. **Olivero OA, Ming JM, Das S, Vazquez IL, Richardson DL, Weston A, Poirier MC.** 2008. Human inter-individual variability in metabolism and genotoxic response to zidovudine. Toxicol Appl Pharmacol **228:**158–164. http://dx.doi.org/10.1016/j.taap.2007.12.005.
34. **Cazzalini O, Lazzè MC, Iamele L, Stivala LA, Bianchi L, Vaghi P, Cornaglia A, Calligaro A, Curti D, Alessandrini A, Prosperi E, Vannini V.** 2001. Early effects of AZT on mitochondrial functions in the absence of mitochondrial DNA depletion in rat myotubes. Biochem Pharmacol **62:**893–902. http://dx.doi.org/10.1016/S0006-2952(01)00713-4.